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(57) Abstract

The present invention provides a method for inhibiting rejection of a transplanted tissue without the use of lethal irradiation. This method comprises grafting bone into a recipient, wherein the bone comprises stromal cells and marrow cells, to induce immunological tolerance of the transplanted tissue by the recipient, thereby inhibiting rejection of the transplanted tissue.

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METHODS FOR INHIBITING REJECTION OF TRANSPLANTED TISSUES BY BONE GRAFTING

The invention disclosed herein was made with Government support under Grants No. 5R29 A1330588 and IR01 A140519, awarded by the National Institutes of Health. The government has certain rights in this invention.

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

The successful induction of transplantation tolerance remains an elusive goal in clinical transplantation. Hematopoietic chimerism in solid organ transplantation is of particular interest because of its potential role as a strategy to induce specific tolerance of the recipient to transplanted tissues (Ildstad, S. T. et al., Nature, 307:168-170 (1984); Sykes, M., et al. Nature Medicine, 3:783-7 (1997); Wekerle, T., et al. Journal of Experimental Medicine, 187:2037-2044 (1998)). Several strategies have been devised to induce tolerance in adult animal models. These have in common the introduction of foreign hematopoietic cells into the recipient after the mature immune system has been depleted by potent immunosuppressive agents (Monaco, A.P. and Wood, M.L. (1970). Studies on heterologous antilymphocyte serum in mice. VII. Optimal cellular antigen for induction of immunological tolerance with ALS, Transplant Proc. 2:489-491; Mayumi, H. and Good, R.A. (1990) Long-lasting skin allograft tolerance in adult mice induced across fully allogeneic (multimajor H-2 plus multiminor histocompatibility) antigen barriers by a tolerance- inducing method using cyclophosphamide, J. Exp. Med. 169(l):213-238; Ildstad, S.T. and Sachs, D.H. (1984) Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts, Nature

307:168-170; Qin, S. et al. (1989) Induction of classical transplantation tolerance in the adult, J. Exp. Med. 169:779-794).

Important disadvantages of these methods is the requirement for rigorous myeloablative procedures and the risk of graft versus host disease (GVHD). These factors have thwarted clinical investigation of these protocols as patient and graft survival rates currently achieved in clinical transplantation make it difficult to subject recipients to more aggressive immunosuppression when the potential for benefit is unknown.

Despite these disadvantages, there is reason to believe that chimerism may play an important role in the induction of acquired tolerance to alloantigen in the adult. Ildstad and co-workers have associated chimerism with the successful induction of transplantation tolerance in the adult rodent model (Ildstad, S.T. and Sachs, D.H. (1984) Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts, Nature 307:168-170). Chimerism has also been linked with prolonged renal allograft survival induced by a combination of donor bone marrow and antithymocyte globulin in non-human primates (Thomas, J.M. et al. (1994) Further studies of veto activity in rhesus monkey bone marrow in relation to allograft tolerance and chimerism, Transplantation, 57(l):101-15). In clinical transplantation, long-term persistent peripheral blood chimerism has been demonstrated, but the immunologic significance of these observations remains unclear (McDaniel, D.O. et al. (1994) Peripheral blood chimerism in renal allograft recipients transfused with donor bone marrow, Transplantation, 57(6):852-856; Suberbielle, C. et al. (1994) Peripheral microchimerism in long-term cadaveric-kidney allograft recipients, Lancet 343:1468-1469; Elwood, E.T. et al. (1997) Microchimerism and rejection in clinical transplantation, Lancet 349:1358-1360). However, the development of protocols that enhance allograft survival utilizing strategies that promote hematopoietic chimerism but do not necessitate extensive conditioning regimens would clearly represent an important advance for clinical transplantation

The bone marrow stromal microenvironment is important for stem cell maturation. Therefore, the establishment of long-term stable peripheral blood chimerism after transfer of bone marrow cells will require a supportive microenvironment (Ishida, T. et al., J. Immunol. 152:3119-27 (1994); Quesenberry, P. J. et al., Blood Cells 20:97-106 (1994); Tomita,Y. et al., Blood 83:939-48 (1994); Down, J. D. et al., Blood 77:661-9 (1991)). This has been approached in the past by myeloablation techniques to "make space" in the recipient bone marrow for donor stem cells (Ildstad, S.T. and Sachs, D.H. (1994) Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts, Nature 307:168-170). Hisha et al. reported improved survival of bone marrow transplant recipients receiving donor-matched bone grafts depleted of bone marrow cells by lethal irradiation (Hisha, H. et al. (1995). Successful bone marrow transplantation by bone grafts in chimeric-resistant combination, Experimental Hematology 23:347-352). These authors attributed the improved survival to the presence of donor-matched stromal cells because (1) stromal cells produce a chemotactic factor that attracts donor-derived hematopoietic stem cells, and (2) stromal cells protect against radioresistant host natural killer cells, cytotoxic T lymphocytes and macrophages. However, Hisha et al. (supra) reported survival of only 50-80% of bone marrow recipients who also received bone-grafts. These authors attributed the limited survival to insufficient reconstitution of the engrafted bones.

An alternate strategy has been to identify the critical cell population ("facilitator cells") that provides this support function, so that it can be augmented in the donor inoculum (Kaufman, C.L. et al. (1994) Phenotypic characterization of a novel bone marrow-derived cell that facilitates engraftment of allogeneic bone marrow stem cells, Blood 84(8):2436-2446). However, this approach remains controversial, and is not widely applied.

Previous work using CTLA4-Ig and anti-gp39 antibodies had demonstrated that they can induce long-term acceptance of cardiac allografts and prolonged survival of fully allogeneic skin grafts (Larsen, C.P. et al. (1996) Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways, Nature 381(6581):434-438). Others have used the immunosuppressant cyclosporin A to achieve tolerance to limb

transplants (Sakai, K. et al. (1993) Vascularized osteochondral allografts in an immunosuppressed rat model: graft modulation and host immune tolerance, Plastic and Reconstructive Surgery 91(4):597-607; Talmor, M. et al. (1995) Bone marrow-derived chimerism in non-irradiated. cyclosporin-treated rats receiving microvascularized limb transplants: evidence for donor-derived dendritic cells in recipient lymphoid tissues, Immunology 86:448-455).

Talmor et al., (supra) transplanted vascularized limb from allogenic donors into recipient rats treated with cyclosporin. Likewise, Sakai et al. (supra) used cyclosporin to treat rats receiving allografts of vascularized knee joint. While these grafts contained bone, the studies were not designed to test whether the presence of bone created tolerance to a transplant nor did the studies compare transplant survival with both cyclosporin and bone to transplant survival with cyclosporin only. In addition, both studies used only vascularized grafts, and therefore did not address whether grafts containing bone could survive or confer a benefit without being vascularized. There still exists a need to provide alternate ways to effect long-term tolerance of transplanted tissues by the host thereby increasing the survival rate of transplantation.

The discovery herein involves transplantation of a donor bone graft with the bone marrow cells intact, preferably under the cover of effective immunosuppression, to allow establishment of the bone graft, but without the requirement for myeloablation in the recipient. This has the advantages of: (1) avoiding the morbidity of myeloablation; and (2) creating "space" in the recipient. This "space" could include important "facilitator cells" without the requirement for laborious isolation techniques as well as a microenvironment of structural and soluble factors which may be important for stem cell maturation. This may be particularly true for xenotransplantation, wherein cytokines and/or growth factors produced by stromal cells from the donor species may have a significant advantage over the recipient's growth factors. For example, human GM-CSF has low activity in promoting development of mouse bone marrow colony development.

The invention involves the discovery that grafting fragments of bone promotes long-term survival of skin grafts of the same donor type as the bone graft, without requiring irradiation of the host. The promotion of long-term graft survival using fragments of whole bone is superior to that obtained using bone marrow.

The effect demonstrated in the transplantation model herein indicates that grafts of whole bone, which include stromal cells, are more effective than bone marrow for inducing long-term immune tolerance. This discovery provides methods that are new and more effective strategies to induce immunological tolerance and to suppress graft rejection.

SUMMARY OF THE INVENTION

The present invention provides a method for inhibiting rejection of a transplanted tissue without myeloablating a recipient of the transplanted tissue. This method comprises grafting bone or fragments thereof into the recipient at a site that vascularizes the bone graft. Grafting can occur before or after transplantation of the tissue. In accordance with the practice of the invention, the bone comprises stromal cells and marrow cells. The presence of bone so grafted results in immunological tolerance of the transplanted tissue by the recipient, thereby inhibiting rejection of the transplanted tissue.

In one embodiment, the bone graft comprises fragments of marrow-containing bone. In another embodiment, the bone comprises a vascularized segment of marrow-containing bone.

In another embodiment rejection of the transplanted tissue is further inhibited by administration of an immunosuppressive agent. Examples of an immunosuppressive agent include, but are not limited to, soluble CTLA4 such as CTLA4 Ig, soluble CD28 such as CD281, soluble B7, such as B71g, soluble gp39, soluble CD40, anti-gp39 antibodies, anti-CD40 antibodies, anti-CD28 antibodies, anti-B7 antibodies, anti-CTLA4 antibodies, cyclosporin, azathioprine, methotrexate, cyclophosphamide, lymphocyte immune globulin, anti-CD3 antibodies, Rho (D) immune globulin, adrenocorticosteroids,

sulfasalzine, FK-506, methoxsalen, mycophenolate mofetil (Cellcept), horse anti-human thymocyte globulin (ATGAM), humanized anti-TAC (HAT), basiliximab (Simulect), rabbit anti-human thymocyte globulin (THYMOGLOBULIN), sirolimus and thalidomide.

In one embodiment, the immunosuppressive agent comprises a first soluble molecule, which prevents an endogenous molecule on a cell selected from the group consisting of gp39 and CD40 from binding its endogenous ligand. In another embodiment, the immunosuppressive agent comprises a second soluble molecule which prevents an endogenous molecule on a cell selected from the group consisting of CTLA4. CD28, and B7 from binding its endogenous ligand. In an alternative embodiment, the immunosuppressive agent comprises a combination of the first soluble ligand and the second soluble ligand. The prevention of these molecules from binding their endogenous ligands blocks two independent signal pathways. The blockage of these two independent signal pathways inhibits the immune responses so that transplant rejection is further inhibited. Further alternatively, multiple immunosuppressive agents can be used in the method of the invention, e.g., a combination of CTLA4Ig, anti-gp39 antibodies and cyclosporin.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a cumulative survival plot showing survival of secondary BALB/c skin grafts in C3H/He mice pre-treated with CTLA41g/MRI only (open triangles), CTLA41g/MR1 and BALB/c bone grafts (closed circles), CTLA41g/MRI and BALB/c bone marrow (closed triangles), and CTLA41g/MRI and C57 bone grafts (open squares).

Figure 2 is a line graph showing percent survival of secondary C57 skin grafts at 50 days after an initial BALB/c heart transplant. Animals in Group I (open squares, received no further treatment), Group 2 (closed circles, received infusion of bone marrow) and Group 3 (closed triangles, received BALB/c bone graft), all promptly rejected secondary skin grafts with all grafts being lost by day 20 post-transplant (closed triangles follow same

trajectory as all four open squares; closed circles follow trajectory of last two open squares). In contrast, the animals in Group 4 (closed squares, received C57 bone graft) accepted secondary C57 skin grafts with all surviving >80 days.

Figure 3 is a line graph showing percent survival of secondary skin grafts in mice pre-treated with CTLA41g/MRI only (closed squares), CTLA41g/MRI and C57 bone graft (closed circles, closed triangles), C57 bone graft only (closed diamonds), or no pre-treatment (open squares). One group (closed triangles) received a BALB/c skin graft; all others received a C57 skin graft.

Figure 4 are graphs of two-color flow cytometry showing bone graft transplantation results in stable multi-lineage hematopoietic chimerism.

Figures 5a-e are graphs and photographs showing transplanted bone grafts populate the recipient's thymus and influence negative selection.

Figures 6a-d are graphs and photographs showing costimulation blockade and bone graft transplantation induce long-term donor-specific unresponsiveness in allogeneic hosts.

Figure 7 includes line graphs showing that bone graft recipients treated with combination costimulatory molecule blockade accept donor specific skin grafts placed 12 hours later.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified. As used in this application, the following words or phrases have the meanings specified.

As used herein, a "bone" or "bone graft" means sufficient bone tissue to include stromal cells and marrow cells. In one embodiment, the bone comprises fragments of bone. The bone grafts of the invention include portions of intact bones from, e.g., animals such as mice or primates. Typically, the bones are femur bones. Other bones may also be used. For use in connection with transplantation in primates such as humans, the graft may consist of a portion of the interior of the bone containing bone fragments or spicules and bone marrow as well as stromal cells. The graft may be removed or scooped, by an instrument such as a curette, from the bone marrow cavity after slicing an end of the bone. The amount of material for use in the graft is such that it is sufficient to induce at least 0.1% hematopoietic chimerism in the subject host. Optimization of the amount of material for the bone graft may be determined empirically.

As used herein, "transplanted tissue" includes autografts, isografts, allografts, and xenografts. Examples of transplanted tissue include, but are not limited to, solid organ grafts such as heart, liver, pancreas or kidney, skin grafts, bone marrow, pancreatic islet cells, cell suspensions, and genetically modified cells.

As used herein, "monoclonal antibodies directed against gp39" includes MR1. Additionally, it includes any antibody molecule, fragment thereof, or recombinant binding protein that recognizes and binds gp39 (gp39 is also known in the literature as the CD40 ligand).

As used herein, "monoclonal antibodies directed against CD40" includes any antibody molecule, fragment thereof, or recombinant binding protein that recognizes and binds CD40.

As used herein, "administering" means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular or subcutaneous administration, or the implantation of a slow-release device such as a miniosmotic pump, to the subject.

As used herein, "pharmaceutically acceptable carrier" includes any material which, when combined with the antibody, retains the antibody's binding specificity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets, including coated tablets and capsules.

Typically, such carriers contain excipients, such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts, thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well-known conventional methods.

As used herein, "B7" includes B7-1 (also called CD80). B7-2 (also called CD86), B7-3), and the B7 family, e.g., a combination of B7-1, B7-2, and/or B7-3.

As used herein, "a soluble ligand which recognizes and binds B7 antigen" includes CTLA4- Ig, CD28-Ig or other soluble forms of CTLA4 and CD28, including recombinant CTLA4 and CD28, and includes any antibody molecule, 6agment thereof or recombinant binding protein that recognizes and binds a B7 antigen.

As used herein, "monoclonal antibodies directed against CTLA4 or CD28" includes any antibody molecule, fragment thereof, or recombinant binding protein that recognizes and binds CTLA4 or CD28.

In order that the invention herein described may be more fully understood, the following description is set forth.

Applicants' discovery is related to a method for inhibiting rejection of a transplanted tissue in a subject. The method can be performed without myeloablating the subject. This method comprises grafting bone or fragments thereof into the subject. Grafting can

occur before or after transplantation of the tissue. In accordance with the practice of the invention, the bone comprises stromal cells and marrow cells. The presence of bone so grafted results in immunological tolerance of the transplanted tissue by the recipient, thereby inhibiting rejection of the transplanted tissue.

Preferably, the bone comprises an intact microenvironment. The microenvironment includes structural and soluble factors which contribute to stem cell maturation. In one embodiment, the bone comprises fragments of bone containing marrow and stromal cells. Examples of sources of bone include, but are not limited to, vertebral bodies, rib, sternum or femur. The bone is taken from a donor antigenically matched with the transplant tissue. Preferably, the bone is syngeneic with the transplant tissue.

In accordance with the invention, the bone may be grafted into the subject before, concurrently with, or after the tissue transplant. When the tissue transplant is grafted into the subject prior to the development of immunotolerance by the bone graft, inhibition of rejection of the tissue transplant can be augmented by the administration of an immunosuppressive agent. Preferably, an immunosuppressive agent is administered prior to, or concurrently with, the grafting of bone to inhibit rejection of the bone graft.

The bone may be grafted into any site in the recipient that is capable of vascularizing the bone graft. Preferably, the bone is grafted into a site having a rich blood supply. Examples of sites suitable for grafting include, but are not limited to omentum or renal capsule. Vascularization and acceptance of the bone graft can be further enhanced by anastomosing one or more blood vessels of the graft with one or more blood vessels of the subject.

The transplanted tissue can be an autograft, isograft, allograft, or xenograft. Examples of transplanted tissue include but are not limited to, solid organ grafts such as heart, pancreas, liver or kidney, skin grafts, bone marrow, pancreatic islet cells, cell suspensions, and genetically modified cells.

In another embodiment, rejection of the transplanted tissue is further inhibited by administration of an immunosuppressive agent. Examples of an immunosuppressive agent include, but are not limited to, soluble CTLA4 such as CTLA4Ig, soluble CD28 such as CD2Ig, soluble B7, such as BB7Ig, soluble gp39, soluble CD40, anti-gp39 antibodies, anti-CD40 antibodies, anti-CD28 antibodies, anti-B7 antibodies, anti-CTLA4 antibodies, cyclosporin, azathioprine, methotrexate, cyclophosphamide, lymphocyte immune globulin, anti-CD3 antibodies, Rho (D) immune globulin, adrenocorticosteroids, sulfasalzine, FK-506. methoxsalen, mycophenolate mofetil (Cellcept), horse anti-human thymocyte globulin (ATGAM), humanized anti-TAC (HAT), basiliximab (Simulect), rabbit anti-human thymocyte globulin (THYMOGLOBULIN), sirolimus and thalidomide.

In one embodiment the immunosuppressive agent comprises a first soluble molecule, such as a soluble ligand, which prevents an endogenous molecule on a cell selected from the group consisting of gp39 and CD40 from binding its endogenous ligand. In another embodiment, the immunosuppressive agent comprises a second soluble molecule, such as a soluble ligand, which prevents an endogenous molecule on a cell selected from the group consisting of CTLA4. CD28, and B7 from binding its endogenous ligand. In an alternative embodiment, the immunosuppressive agent comprises a combination of the first soluble ligand and the second soluble ligand. The prevention of these molecules from binding their endogenous ligands blocks two independent signal pathways. The blockage of these two independent signal pathways inhibits the immune responses so that transplant rejection is further inhibited. Further alternatively, multiple immunosuppressive agents can be used in the method of the invention, e.g., a combination of CTLA4Ig, anti-gp39 antibodies and cyclosporin.

The first soluble molecule, which prevents an endogenous molecule on a cell selected from the group consisting of gp39 and CD40 from binding its endogenous ligand, can be a soluble ligand which recognizes and binds a gp39 antigen on gp39-positive cells or CD40 antigen on CD40-positive cells. The soluble ligand can be a monoclonal antibody directed against gp39 or against CD40.

The second soluble molecule, which prevents an endogenous molecule on a cell selected from the group consisting of CTLA4, CD28 and B7 from binding its endogenous ligand, can be a soluble ligand which recognizes and binds a CD28 antigen on CD28-positive cells or a B7 antigen on B7 positive cells. The soluble ligand can be CTLA4Ig, CD28Ig, B7Ig or other soluble form of CTLA4, CD28 or B7, including recombinant CTLA4, CD28 or B7. Alternatively, the soluble ligand can be a monoclonal antibody or fragment thereof directed against a B7 antigen, CTLA4 or CD28.

Immunosuppressive agents may be administered concurrently with the tissue transplant, before the tissue transplant, after the tissue transplant, concurrently with the bone graft, before the bone graft, or after the bone graft. Immunosuppressive agents may be administered by oral means, transdermal means, intravenous means, intramuscular means, intraperitoneal, or by subcutaneous administration. The most effective mode of administration and dosage regimen for the molecules of the present invention depends upon the location of the tissue or disease being treated, the severity and course of the medical disorder, the subject's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the molecules should be titrated to the individual subject.

Similarly, the amount of bone graft required to achieve inhibition of transplant rejection may be determined by routine experimentation and optimized empirically.

By way of example, the interrelationship of dosages for animals of various sizes and species and humans based on mg/m 2 of surface area is described by Freireich, E.J., et al. Cancer Chemother., Rep. 50 (4): 219-244 (1966). Adjustments in the dosage regimen may be made to optimize suppression of the immune and inflammatory response resulting in graft rejection, e.g., doses may be divided and administered on a daily basis or the dose reduced proportionally depending upon the situation (e.g., several divided doses may be administered daily or proportionally reduced depending on the specific therapeutic situation).

It would be clear that the dose of the immunosuppressive agent required to achieve an appropriate clinical outcome may be further reduced with schedule optimization.

The present invention also provides pharmaceutical compositions useful in inhibiting graft rejection. These compositions comprise an effective amount of fragments of bone and an acceptable carrier. In one embodiment, the composition further comprises an immunosuppressive agent.

ADVANTAGES OF THE INVENTION: The experiments described herein show that transplant tolerance can be achieved with the grafting of bone and without the use of myeloablation (e.g., irradiation) of the recipient. The experiment also demonstrates the generation of stable multi-lineage hematopoietic chimerism resulting from bone graft transplantation. The methods of the invention offer the advantages of avoiding the morbidity associated with myeloablation and avoiding laborious isolation techniques to obtain facilitator cells. The bone grafts of the invention provide a supportive microenvironment of structural and soluble factors which may be important for stem cell maturation.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLE 1

This example shows that the concomitant placement of a donor bone graft at the time of heart transplantation induces a long-term state of specific transplantation tolerance towards the donor antigens.

Methods

Male C3H/He (H2 k), BALB/c (H2 d), and C57BL/6 (H2 b) mice (Jackson Laboratory, Bar Harbor, ME) were used at 8-12 weeks of age. Recipients were all C3H mice.

Transplantation Procedures

Primarily vascularized heterotopic heart transplantation was performed using microsurgical techniques (Corry, R.J. et al. (1973) Primarily vascularized allografts of hearts in mice: The role of H-2D, H-2K, and non H-2 antigens in rejection, Transplantation 16(4):343-350). Rejection was defined by the loss of palpable cardiac contractions with confirmation at laparotomy by direct visualization.

Full thickness ear skin was grafted onto the posterior lateral thoracic wall of recipient mice. The grafts were then followed by daily visual inspection. Rejection was defined as the complete loss of visible epidermal graft tissue. BALB/c skin grafts were used for Part A of the experiment. For Part B, C57 skin grafts were used.

The bones (one femur and one tibia) were sectioned into small pieces and were engrafted under the recipient renal capsules. The bone grafts consisted of fragments of whole bone, which includes cortex and marrow. The bone marrow in turn contained both stromal and hematopoietic elements.

Preparation of Bone Marrow Cells

Bone marrow cells were flushed from the femurs and tibias of donor mice using a 27-gauge needle. The cells were prepared for injection by passage through a 70 um cell strainer (Becton Dickinson) and by red blood cell lysis with Tris-buffered ammonium chloride. Bone marrow cells (2x10 7 cells/dose) in sterile phosphate-buffered saline were administered intravenously to cardiac allograft recipients at the completion of the transplant procedure.

Treatment Protocols

3CH/He heart transplant or heart/bone transplant recipients were treated intravenously with 500 μg each of human CTLA4-Ig and MRI (hamster anti-mouse gp39 monoclonal antibody) at the time of transplantation of a BALB/c heart and intraperitoneally on days 2, 4 and 6 after transplantation. Group I received no further treatment. Group 2 received an infusion of 2x I 07 BALB/c bone marrow cells. Group 3 received a BALB/c bone graft placed under the right kidney capsule while Group 4 received a C57 bone graft at the time of heart transplantation.

Heart graft survival was followed for the ensuing 50 days all recipients accepted their initial BALB/c heart graft. At 50 days after the time of heart transplantation, the animals were rechallenged with either a BALB/c (Part A) or C57 skin (Part B) graft.

Results

The results of Part A (BALB/c skin grafts) are shown in Figure 1. The animals in Group I which received no bone marrow or bone graft preparation all rejected their secondary BALB/c skin grafts with a median survival time (MST) of 51 days (n=7). Animals in Group 2, which received an infusion of BALB/c bone marrow cells at the time of heart transplantation, demonstrated a MST of 78 days with only one BALB/c skin graft surviving for >100 days (n=8). In contrast, animals in Group 3, which received a BALB/c bone graft at the time of heart transplantation, demonstrated markedly prolonged survival of secondary BALB/c skin grafts placed 50 days after the original heart transplant and bone grafting (n=8). The MST in this group was >104 days, furthermore the long-term surviving skin grafts in these animals were large, supple, and healthy in appearance in contrast to the single long-term survivor in Group 2. Finally, the specificity of the tolerance induced by bone grafting was demonstrated by Group 4, which received a C57 bone graft at the time of placement of BALB/c heart graft. Upon rechallenging with secondary BALB/c skin grafts at 50 days, these animals uniformly rejected the BALB/c skin grafts with a MST of 28 days (n=6).

In Part B of the experiment, additional members of Groups 1, 2, 3 and 4 were challenged with a secondary C57 skin graft at 50 days after the initial BALB/c heart transplant (Figure 2). Animals in Groups 1 (n=4), 2 (n=3), and 3 (n=4), all promptly rejected secondary skin grafts with all grafts being lost by day 20 post-transplant. In contrast, the animals in Group 4 (n=3), which had received CTLA4-Ig, MRI, a BALB/c heart transplant, and a C57 bone graft at day 0, accepted secondary C57 skin grafts with all surviving >80 days. These results demonstrate that the bone graft was the primary toleragen in this experimental system and that the tolerance induced is directed exclusively towards the bone graft donor type.

EXAMPLE 2

This example shows that a bone graft can induce donor-specific tolerance to a subsequent skin graft.

Methods

Male C3H/He (H2 k), BALB/c (H2 d), and C57BL/6 (H2 b) mice (Jackson Laboratory, Bar Harbor, ME) were used at 8-12 weeks of age.

Transplantation Procedures

Full thickness ear skin was grafted onto the posterior lateral thoracic wall of recipient mice (Billingham, R.E. and Medawar, P.B. (1951) Technique of free skin grafting in mammals, J. Exp. Biol. 28:385). The grafts were then followed by daily visual inspection. Rejection was defined as the complete loss of visible epidermal graft tissue.

The bones (one femur and one tibia) were sectioned into small pieces and were engrafted unilaterally under the recipient renal capsule.

Preparation of Bone Marrow Cells

Bone marrow cells were flushed from the femurs and tibias of donor mice using a 27-gauge needle. The cells were prepared for injection by passage through a 70-um cell strainer (Becton Dickinson) and by red blood cell lysis with Tris-buffered ammonium chloride. Bone marrow cells (2xlO 7 cells/dose) in sterile phosphate-buffered saline were administered intravenously.

Treatment Protocols

C3H/He male mice were divided into five experimental groups. Group I received no pretreatment. Group 2 was treated intraperitoneally with 500 μg each of human CTLA4 Ig and MRI (hamster anti-mouse gp39 monoclonal antibody) on days 1, 2. 4, and 6.

Group 3 received a C57 bone graft under the right kidney capsule. Group 4 received a C57 bone graft under the right kidney capsule and was treated intraperitoneally with 500 μg each of human CTLA4-Ig and MRI (hamster anti-mouse gp39 monoclonal antibody) on days 1, 2, 4, and 6. Group 5 also received a C57 bone graft under the right kidney capsule and was treated intraperitoneally with 500 μg each of human CTLA4-Ig and MRI on days 0, 2, 4, and 6. Fifty days later the mice in groups 1-4 received a C57 skin graft and no additional treatment. The mice in group 5 received a Balb/c skin graft and no additional treatment.

Results

The results of the experiment are shown in Figure 3. Untreated mice (n=4) or mice that received only CTLA4-Ig/MRI (n=5) or only a bone graft (n=4) 50 days previously promptly rejected C57 skin grafts. Mice which received both a C57 bone graft and CTLA4-Ig/MRI pretreatment accepted a C57 skin graft (n=10). These results demonstrate that both the bone graft and peri-bone grafting immunosuppression with CTLA4-Ig/MRI contribute to the induction of tolerance. The complete immunologic

recovery and donor specificity of the tolerance induction were demonstrated by group five, which promptly rejected BALB/c skin grafts (n=4).

EXAMPLE 3

This example demonstrates the induction of hematopoietic chimerism using bone graft transplantation

Methods

In this protocol, fragments of femur from B6 (Ly5b) donors were transplanted under the kidney capsule of congenic B6.SJL (Ly5a) recipients.

Bone grafting. Femurs were harvested from donor mice and fragments of one femur were transplanted under the kidney capsule of recipient mice.

Flow cytometric analysis. Analysis of peripheral blood and spleen were carried out using fluorochrome-conjugated antibodies (anti-Ly5.1, anti-Ly5.2, anti-CD4, anti-CD11c, anti-$H2K^d$, anti-$I\text{-}A^d$, anti-Vβ11, Pharmingen). Immunohistochemistry. Cryosections (7μm) were prepared and stained with biotinylated anti-$I\text{-}A^d$ (Pharmingen) followed by ABC complex (Vector). Peroxidase activity was visualized using DAB substrate (Pierce). Cytospin slides of bone graft cultures were stained with biotinylated primary antibodies (anti-$H2K^b$, anti-$H2K^k$, anti-$I\text{-}A^b$, anti-$I\text{-}A^k$) followed by streptavidin-FITC or using FITC conjugated mAbs (Pharmingen).

Skin grafting. Full thickness skin grafts (~ 1 cm^2) were transplanted on the thorax of recipient mice and secured with a Bandaid® for 7 days.

Mixed leukocyte reactions. Splenic T cells and DC were prepared and used in mixed leukocyte reactions essentially as described [21].

Results

Two weeks after bone graft transplantation few if any donor Ly5b cells were detectable in the peripheral blood of the B6.SJL recipients (Fig. 4). However, by six weeks, multi-lineage (lymphocyte, monocyte, and granulocyte) was readily demonstrable in the peripheral blood. (Ildstad, S. T. et al., Nature, 307:168-170 (1984); Sykes, M., et al. Nature Medicine, 3:783-7 (1997); Wekerle, T., et al. Journal of Experimental Medicine, 187:2037-2044 (1998)). Similar levels of donor cells were also detected at 10, 20, and 30 weeks after transplantation indicating that bone graft transplantation had resulted in engraftment of long-term repopulating pluripotent stem cells.

Figure 4 shows bone graft transplantation results in stable multi-lineage hematopoietic chimerism. Peripheral blood WBC (Ly5+) were analyzed for the % of donor-derived (Ly5b+) cells within the granulocyte, monocyte and lymphocyte compartments using two color flow cytometry at 2, 10, and 30 weeks after bone graft transplantation. Results shown are from a single representative mouse of eight from two separate experiments.

Further, at 30 weeks the viability of the bone graft was established by propagating donor derived cells from the bone grafts *ex vivo* in media containing recombinant granulocyte-macrophage colony stimulating factor (GM-CSF). These results indicate that in the absence of an immunologic barrier, transplantation of bone marrow in the form of a bone graft can produce long-term hematopoietic chimerism.

EXAMPLE 4

In this example and Examples 5-7, the bone graft for transplantation consisted of crushed mouse femur containing bone fragments, bone marrow and stromal cells.

In this example, hematopoietic progenitors from a transplanted bone graft were tested to determine whether they could populate the recipient thymus and influence negative

selection of T cells. B6SCID mice were transplanted with bone grafts from B6 (H-2^b) or B6CF1 (H-2^{bxd}, B6C) nude donor mice. Nude mice fail to develop T cells because of a thymic epithelial defect. In contrast, bone marrow cells from SCID mice fail to generate T cells because they lack a functional DNA-dependant kinase gene that is essential for T cell receptor arrangement. After 12 weeks the peripheral blood CD4+ T cells which developed in these mice were examined by flow cytometry. As expected, SCID mice that did not receive bone grafts failed to develop T cells, even after 20 weeks. In contrast, recipients of B6 of B6CF1 nude bone grafts slowly developed T cells.

The results of these experiments are shown in Figure 5a-e. In recipients of B6CF1 bone grafts, the T cells uniformly expressed H-$2K^d$, confirming the donor origin (F1) of the T cell progenitors (Figure 5a). The percentage of CD4+ cells that expressed T cell receptor containing Vβ11 is shown in Figure 5b. In Balb/C mice, Vβ11 bearing T cells are eliminated in the thymus because of their high affinity for an endogenous retroviral superantigen presented by I-E MHC class II molecules. B6 mice lack I-E molecules and use Vβ11 on approximately 4 to 5% of Cd4+ T cells. Vβ11+ cells comprised 4 to 5% of the CD4 compartment of recipients of B6 bone grafts and <1% in recipients of B6CF1 bone grafts (Figure 5b).

Immunohistochemical analysis confirmed that the thymus of B6CF1 bone graft recipients contained donor derived MHC class II+ cells (Figure 5c). The thymus from recipients of B6 (Figure 5c, left) or B6CF1 (Figure 5c, right) bone grafts were stained for the presence of Ia^d- bearing cells.

Similarly, flow cytometric analysis revealed that 5 to 10% of CD11c+ splenic dendritic cells were of donor origin (Figure 5d). Transient adherent splenocytes from bone graft recipients were labeled for expression of CD11c and Ia^d. 5 to 10% of CD11c+ cells from B6CF1 bone grafts were Ia^d+ indicating their donor origin.

The response of Balb/C and C3H/HeJ skin grafts was also determined. Fifteen weeks after bone grafting, recipients were challenged with Balb/C and C3H skin grafts.

Recipients of B6CF1 nude bone grafts that developed T cell indefinitely (>50 days) accepted Balb/C skin grafts without evidence of rejection, but promptly rejected C3H skin grafts as shown in Figure 5e. In contrast, recipients of B6 nude bone grafts rejected skin grafts from either donor. Similar results were obtained in three independent experiments. These results demonstrate the I-E bearing bone graft-derived cells populate the recipient thymus in sufficient numbers to shape the selection of the T cell repertoire and confer robust donor-specific tolerance.

EXAMPLE 5

Experiments were performed to determine whether transplantation of a bone graft could induce hematopoietic chimerism and transplantation unresponsiveness across an allogeneic barrier in immunocompetent mice. It was previously reported that blockade of the CD40 and CD28 pathways (costimulatory molecule blockade) can produce indefinite survival of cardiac allografts, but does not confer transplantation tolerance to secondary skin grafts (Larsen, C. P. et al., Nature 381:434-438 (1996)).

It was reasoned that short-term costimulatory molecule blockade might prevent rejection of an allogeneic bone graft and allow the donor hematopoietic cells to engraft and promote donor-specific unresponsiveness. Therefore, bone grafts from B6 mice were transplanted to C3H recipients which were treated with anti-CD40L antibody and CTLA4-Ig for six days. After 12 weeks, the ability of the experimental and control mice were assessed to respond to donor and third party alloantigen *in vitro* and *in vivo*.

Results

T cells from naive C3H mice generated strong proliferative responses when challenged with B6 or Balb/c dendritic cells. Similarly, mice that received costimulation blockade alone or a B6 bone graft and no costimulation blockade responded vigorously to both B6 and Balb/c stimulators. In contrast, 4 of 5 mice that received a B6 bone graft and

costimulation blockade 12 weeks earlier displayed significantly diminished responses to B6 DC, but retained normal reactivity to Balb/c dendritic cells (Fig. 6a).

EXAMPLE 6

In this example, the ability to induce long term donor-specific unresponsiveness in allogeneic hosts using costimulation blockade and bone graft transplantation was determined.

Bone grafts from recipient mice were harvested and cultured in media containing GMCSF to determine their viability. Groups of C3H mice received either no treatment, a B6 bone graft alone, costimulation blockade and no bone graft, or costimulation blockade and a B6 bone graft. Twelve weeks later responsiveness to donor (B6) and third-party (Balb/C) was tested *in vitro* and *in vivo*. Figure 6 shows the result of these experiments.

Costimulation blockade and bone graft transplantation induce donor-specific hyporesponsiveness in a mixed leukocyte reaction. (Figure 6a-responses to B6 (donor, filled squares); Balb/C (third party, open triangles); or C3H (syngeneic, open circles)). Bone grafts from recipients that were not treated with costimulatory molecule blockade contains no viable hematopoietic progenitors and could not be propagated in culture demonstrating that costimulation blockade protects the hematopoietic potential of bone grafts transplanted to allogeneic hosts (Figure 6b, bone marrow culture from a normal B6 mouse propagated in media containing GM-CSF (150 U/ml, left); nonviable B6 bone graft culture from an untreated C3H recipient (center); B6 bone graft culture from a C3H recipient treated with costimulation blockade 12 weeks after transplantation (right)).

In contrast, 4 of 5 bone grafts from treated mice generated bone marrow colony units. Bone marrow cultures were propagated in GM-CSF and labeled with anti-Ia^b monoclonal antibodies. Interestingly, the single bone graft without viable cells was from the same animal that failed to display donor-specific hyporesponsiveness. To determine the origin of the hematopoietic cells that propagated *in vitro*, these cells were analyzed for

expression of donor and recipient MHC molecules in cytospin preparations. While the majority of cells propagated from the bone grafts were of recipient origin (H-2K^k), donor MHC Class I+ and class II+ cells were clearly present (Figure 6c). (Figure 6c; normal B6 bone marrow (left): normal C3H bone marrow (center) or a B6 bone graft culture from a C3H recipients treated with costimulation blockade 12 weeks after transplantation).

In addition, costimulation blockade was used with bone graft transplantation to determine the effects on donor-specific skin graft acceptance. Groups of C3H mice received either no treatment (Figure 6d, filled diamond); a B6 bone graft alone (Figure 6d, open triangle); costimulation blockade and no bone graft (Figure 6d, open circle); or costimulation blockade graft and a B6 bone graft (Figure 6d, filled square). Twelve weeks later recipients were challenged with third-party (Balb/C, Figure 6d, left) or donor-specific (B6, Figure 6d, right) skin grafts. In a similar experiment 80% of mice receiving costimulation blockade and a donor bone graft accepted donor skin grafts for >100 days.

These results demonstrate that costimulation blockade preserved the hematopoietic potential of the transplanted bone marrow microenvironment.

EXAMPLE 7

To determine whether transplantation of a bone graft and perioperative CD40/CD28 blockade is able to confer durable donor-specific unresponsiveness *in vivo*, mice were challenged 12 weeks after this induction protocol with donor (B6) or third-party (Balb/c) skin grafts (Fig. 7). C3H mice that received a B6 bone graft in the absence of costimulatory molecule blockade promptly rejected both donor-specific and third party skin grafts. Similarly, mice that had been treated with costimulatory molecule blockade alone also promptly rejected B6 and Balb/c skin grafts, indicating no residual drug effect 12 weeks after administration. In contrast, mice that received a B6 bone graft and costimulatory molecule blockade accepted donor-specific B6 skin grafts, while promptly rejecting third party Balb/c grafts.

These experiments demonstrate a novel strategy to induce hematopoietic chimerism without the need for irradiation or a myeloreductive preparatory regimen. This approach may also provide an effective means to induce donor-specific transplantation tolerance to solid organ transplants. Furthermore, transplantation of the intact bone marrow microenvironment may provide distinct advantages in xenotransplantation, where the species selectivity of critical hematopoietic growth factors appears to be an important nonimmune barrier to long-term engraftment of xenogeneic HSCs (Nikolic, B. et al., Transplantation 65:1216-1224 (1998); Gritsch, H. A. and Sykes, M. Xenotransplantation 3:312-320 (1996); Warrens, A. N. et al., Transplantation 66:252-259 (1998)). However, in xenogeneic and certain allogeneic systems adjunctive immunosuppressive approaches may be necessary to adequately protect the bone graft (e.g., inhibition of NK cell mediated bone marrow rejection, Yu, Y. Y. and Bennett, M. Annu Rev Immunol 10:189-213 (1992)). Bone graft transplantation may also have potential to restore missing cell populations or immunologic functions in hereditary immunodeficiency syndromes (e.g. Bruton's agammaglobulinemia) or to treat hematopoietic diseases characterized by failure of the bone marrow microenvironment such as myelofibrosis without the need for myeloreductive conditioning.

What is claimed is:

1. A method for inhibiting rejection of a transplanted tissue in a subject comprising grafting bone into the subject at a site that vascularizes the bone graft, wherein the bone so grafted comprises stromal cells and marrow cells, the bone so grafted inducing immunological tolerance of the transplanted tissue by the subject thereby inhibiting rejection of the transplanted tissue.

2. The method of claim 1, wherein the bone is grafted before the tissue transplant.

3. The method of claim 1, wherein the bone is grafted after the tissue transplant.

4. The method of claim 1, wherein the bone is grafted concurrently with the tissue transplant.

5. The method of claim 1, wherein the bone comprises fragments of bone sufficient to produce stable hematopoietic chimerism in the subject.

6. The method of claim 1, wherein the bone is grafted into omentum or a renal capsule of the subject.

7. The method of claim 1, wherein the transplanted tissue is heart, liver, kidney, skin, bone marrow, pancreas or pancreatic islets.

8. The method of claim 1 further comprising the administration of an immunosuppressive agent.

9. The method of claim 8 wherein the immunosuppressive agent is administered before the bone graft.

10. The method of claim 8, wherein the immunosuppressive agent is administered after the bone graft.

11. The method of claim 1, wherein one or more blood vessels of the graft are anastomosed with one or more blood vessels of the subject.

12. The method of claim 1, wherein tissue is an autograft, isograft, allograft or xenograft.

13. The method of claim 8, wherein the immunosuppressive agent is administered concurrently with the bone graft.

14. The method of claim 8, wherein the immunosuppressive agent is administered before the tissue transplant.

15. The method of claim 8, wherein the immunosuppressive agent is administered after the tissue transplant.

16. The method of claim 8, wherein the immunosuppressive agent is administered concurrently with the tissue transplant.

17. The method of claim 8, wherein the administration of an immunosuppressive agent comprises administering to the subject an effective amount of a combination of a first soluble ligand which recognizes and binds the B7antigen on B7-positive cells and a second soluble ligand which recognizes and binds the gp39 antigen on gp39-positive cells, the binding of B7-positive cells with the first soluble ligand and gp39-positive cells with the second soluble ligand thereby disrupting endogenous CTLA4-, CD28-, and CD40-positive cell interactions with B7-positive cells and gp39-positive cells so that transplant rejection is further inhibited.

18. The method of claim 15, wherein the first soluble ligand is CTLA41g.

19. The method of claim 15, wherein the first soluble ligand is soluble CD28.

20. The method of claim 15, wherein the second soluble ligand is an antibody directed against gp39.

21. The method of claim 15, wherein the first soluble ligand is a monoclonal antibody reactive antigen.

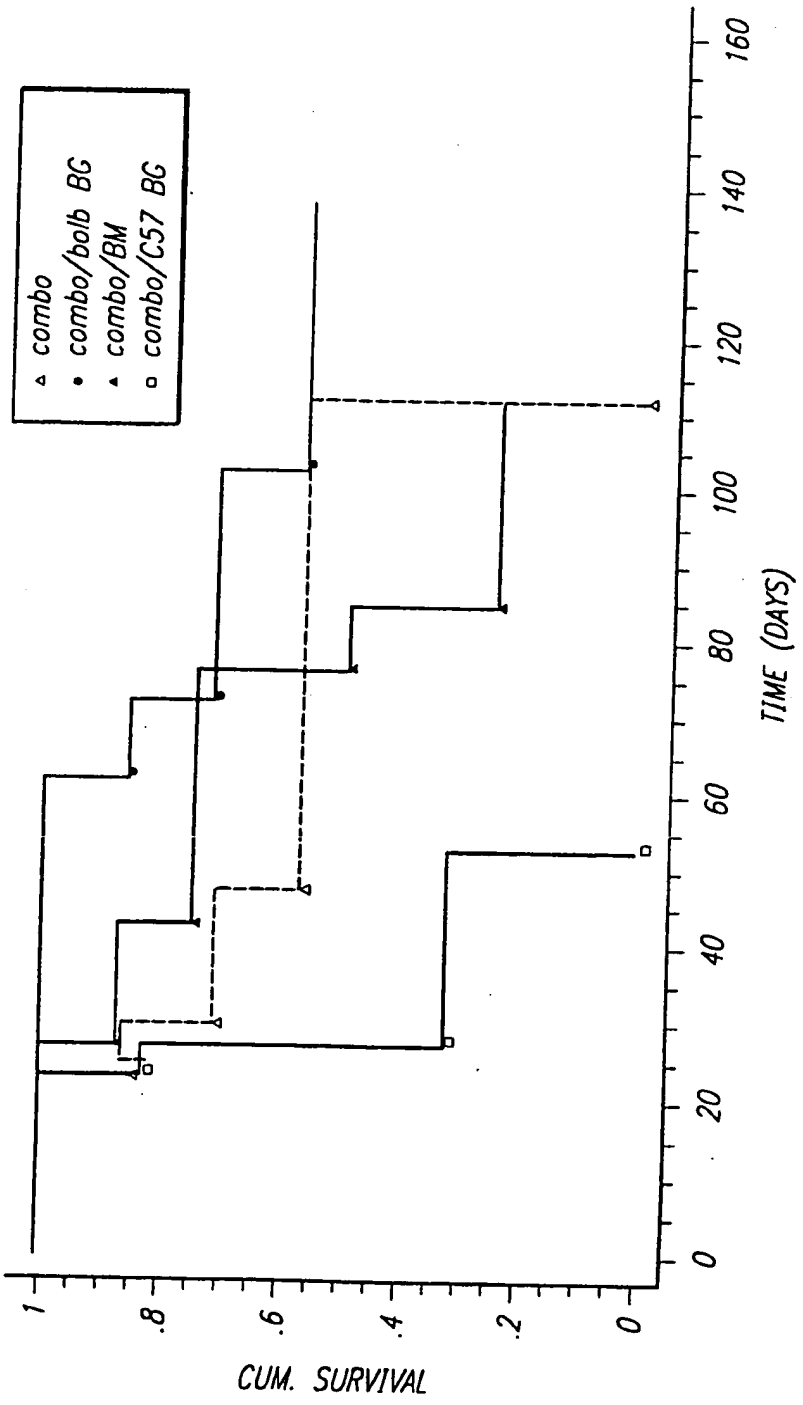
22. The method of claim 19, wherein the antibody is anti-BBI monoclonal antibody.

23. The method of claim 15, wherein the second soluble ligand is a soluble CD40 molecule.

24. The method of claim 8, wherein the immunosuppressive agent is selected from the group consisting of cyclosporin, azathioprine, methotrexate, cyclophosphamide, lymphocyte immune globulin, anti-CD3 antibodies, anti-CD40 antibodies, Rho (D) immune globulin, adrenocorticostero ids, sulfasalzine, FK-506, methoxsalen, mycophenolate mofetil, horse anti-human thymocyte globulin (ATGAM), humanized anti-TAC (HAT), basiliximab, rabbit anti-human thymocyte globulin, sirolimus and thalidomide.

25. A method of producing stable hematopoietic chimerism in a subject comprising grafting marrow-containing bone and stromal cells into a site in said subject that vascularizes the bone graft.

FIG. 1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combo/balb BG
combo/BM
combo/C57 BG
TIME (DAYS)
0
20
40
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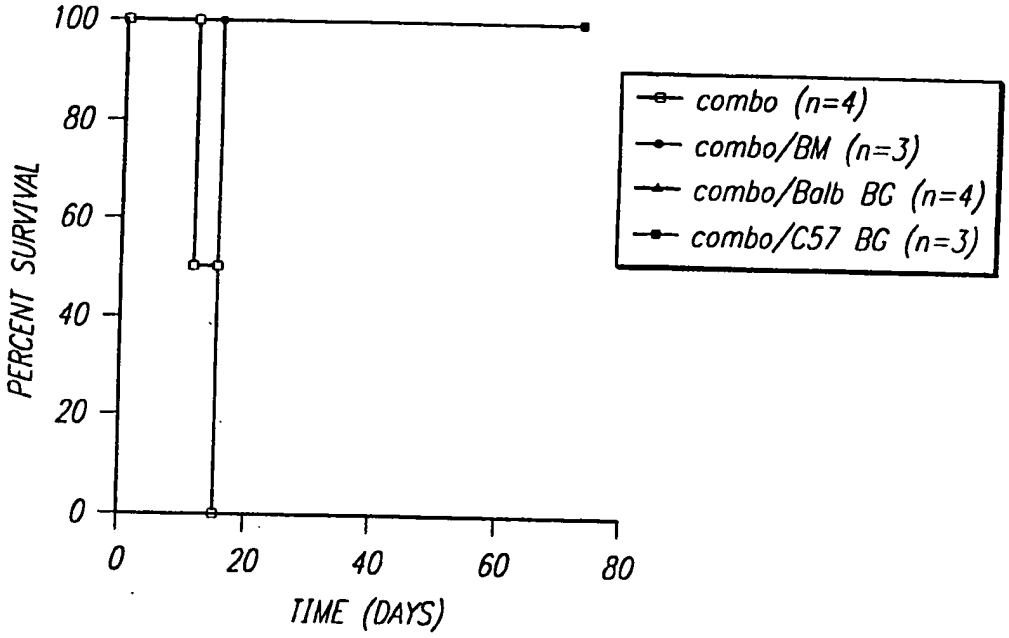
80
100
120
140
160
CUM. SURVIVAL
1
.8
.6
.4
.2
0

FIG. 2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80
60
40
20
0
PERCENT SURVIVAL
0
20
40
60
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TIME (DAYS)
combo (n=4)
combo/BM (n=3)
combo/Balb BG (n=4)
combo/C57 BG (n=3)

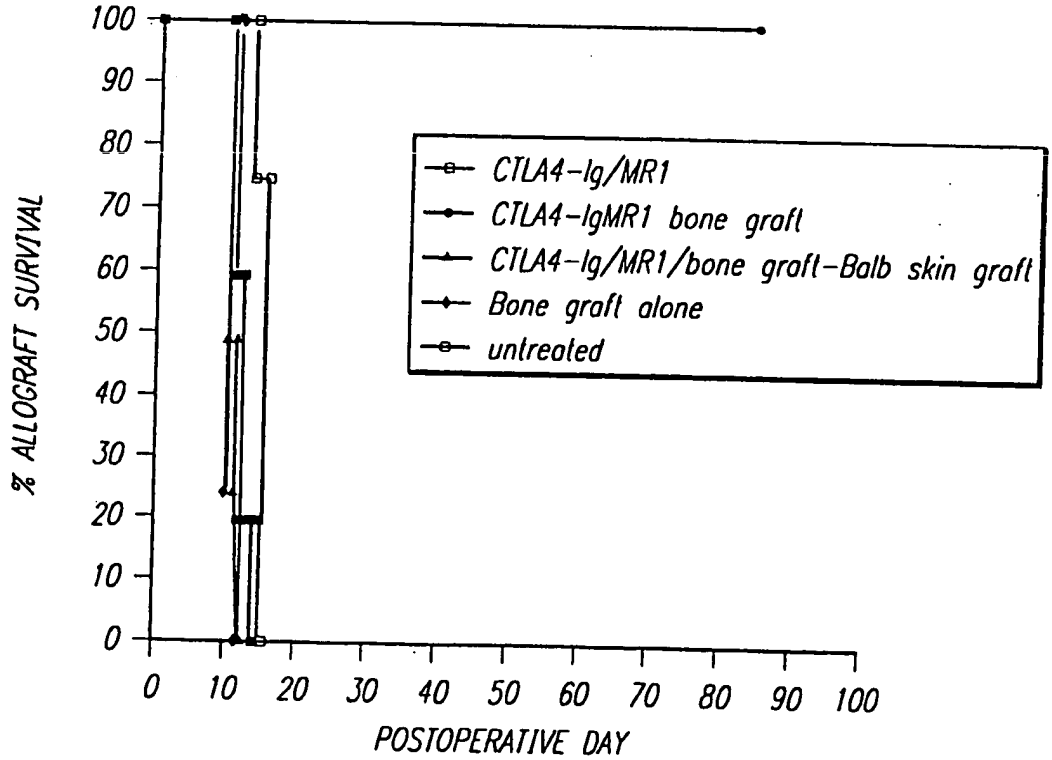
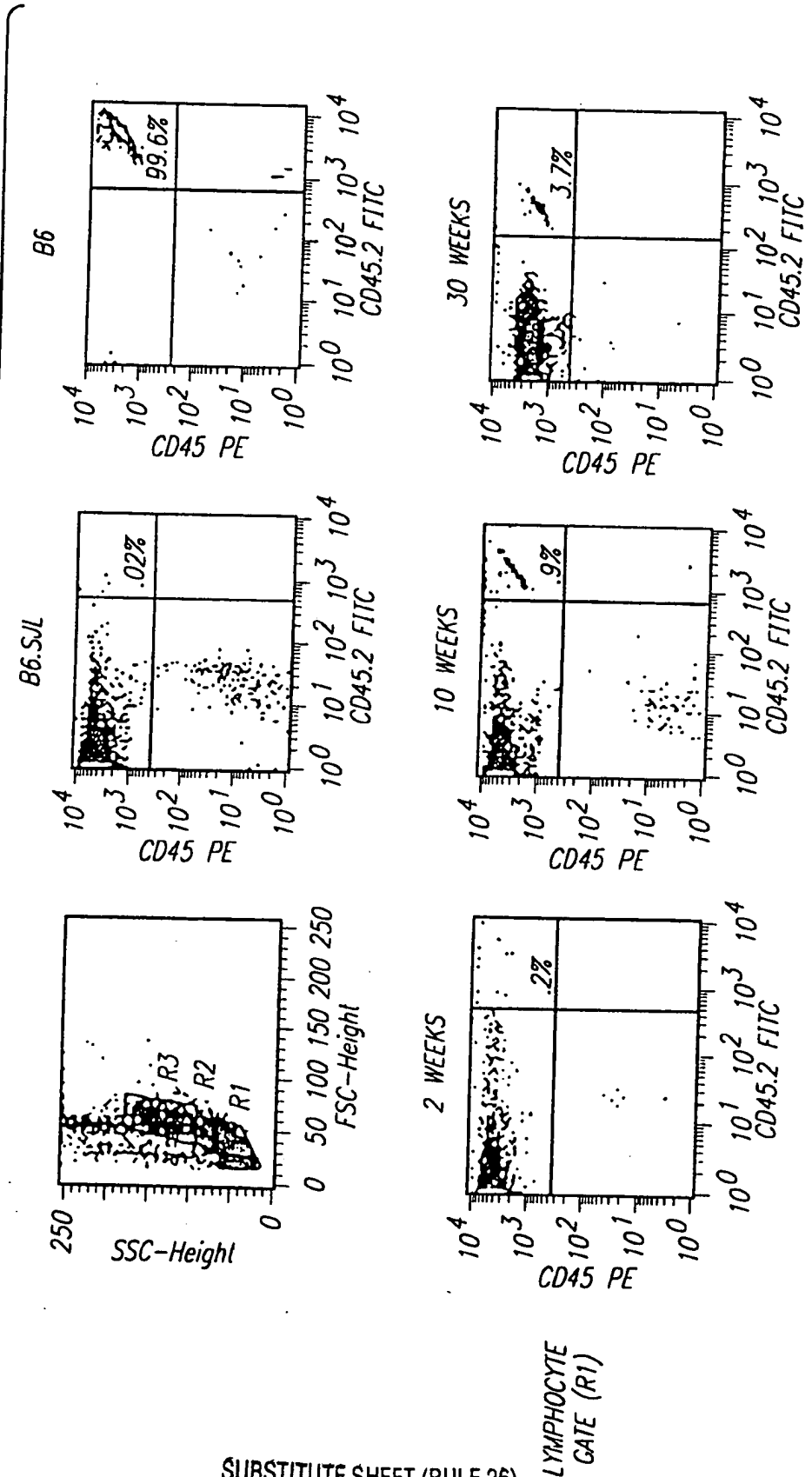

100
90
80
70
60
50
40
30
20
10
0
% ALLOGRAFT SURVIVAL
0
10
20
30
40
50
60
70
80
90
100
POSTOPERATIVE DAY
CTLA4-Ig/MR1
CTLA4-IgMR1 bone graft
CTLA4-Ig/MR1/bone graft-Balb skin graft
Bone graft alone
untreated

FIG. 3

FIG. 4-1


B6
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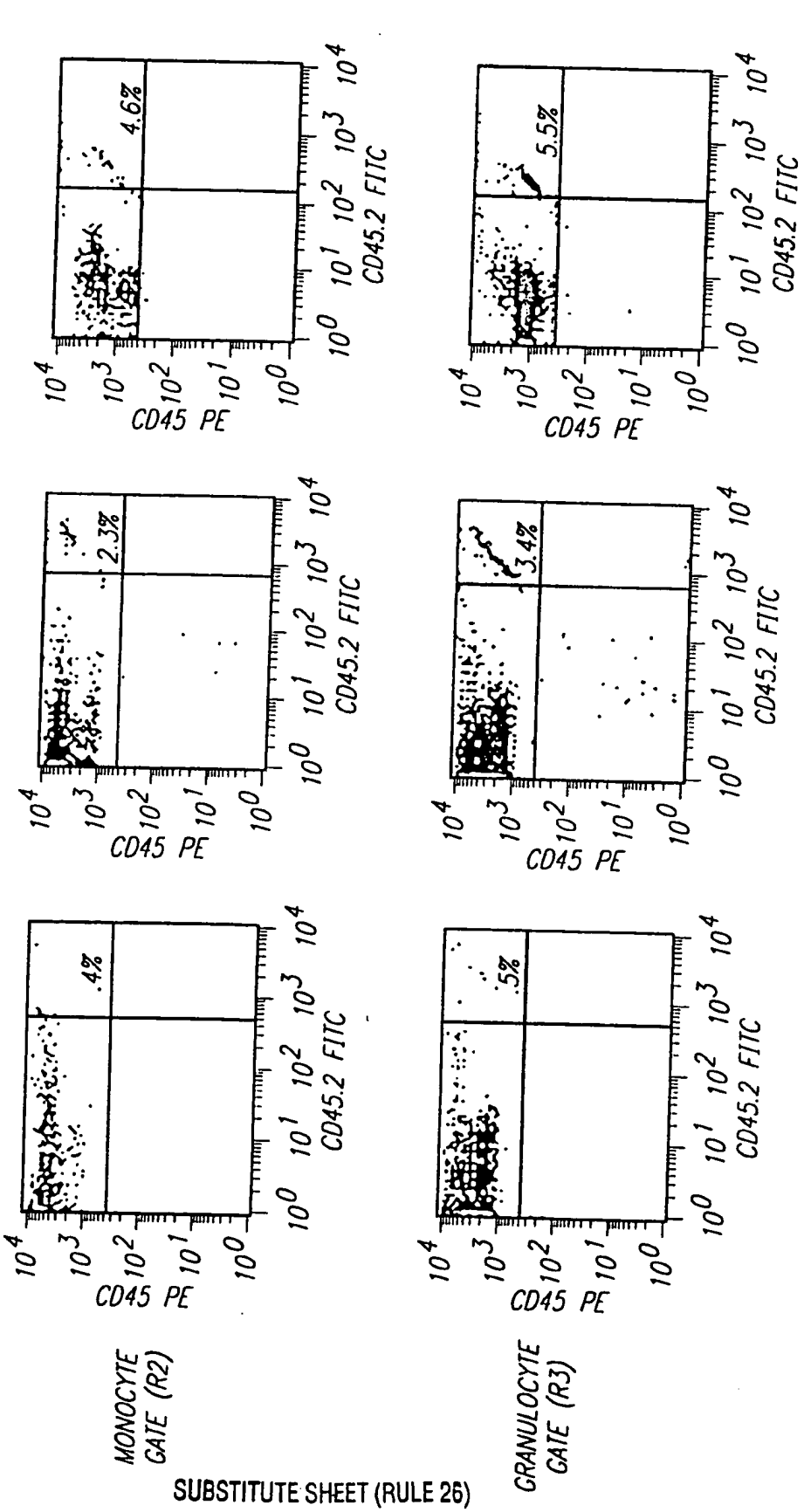
CD45 PE
CD45.2 FITC
30 WEEKS
3.7%
B6.SJL
.02%
10 WEEKS
.9%
2 WEEKS
.2%
R3
R2
R1
SSC-Height
FSC-Height
LYMPHOCYTE GATE (R1)

FIG. 4-2


4.6%
5.5%
2.3%
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CD45 PE
CD45.2 FITC
MONOCYTE GATE (R2)
GRANULOCYTE GATE (R3)

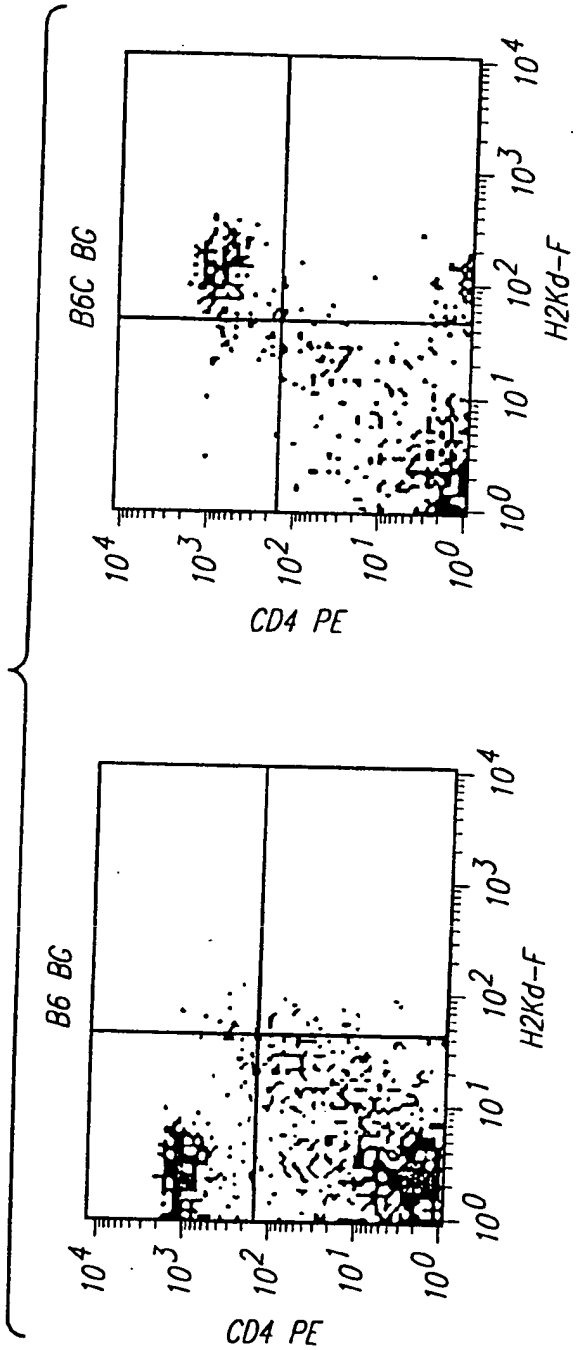

B6C BG
H2Kd-F
CD4 PE
B6 BG
H2Kd-F
CD4 PE

FIG. 5A

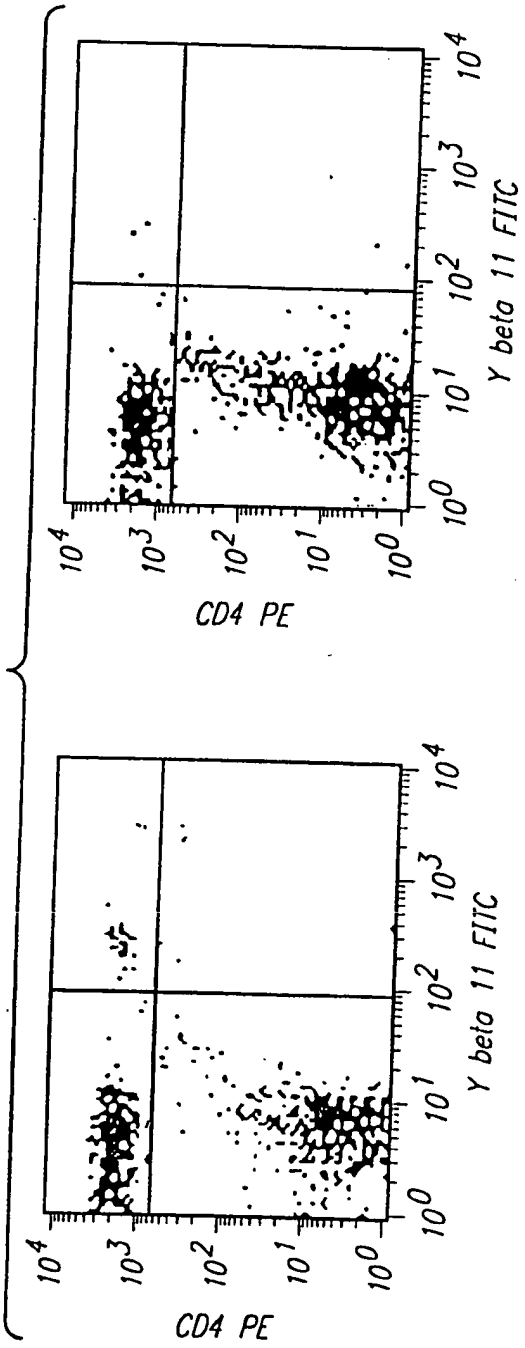

Y beta 11 FITC
CD4 PE
Y beta 11 FITC
CD4 PE

FIG. 5B

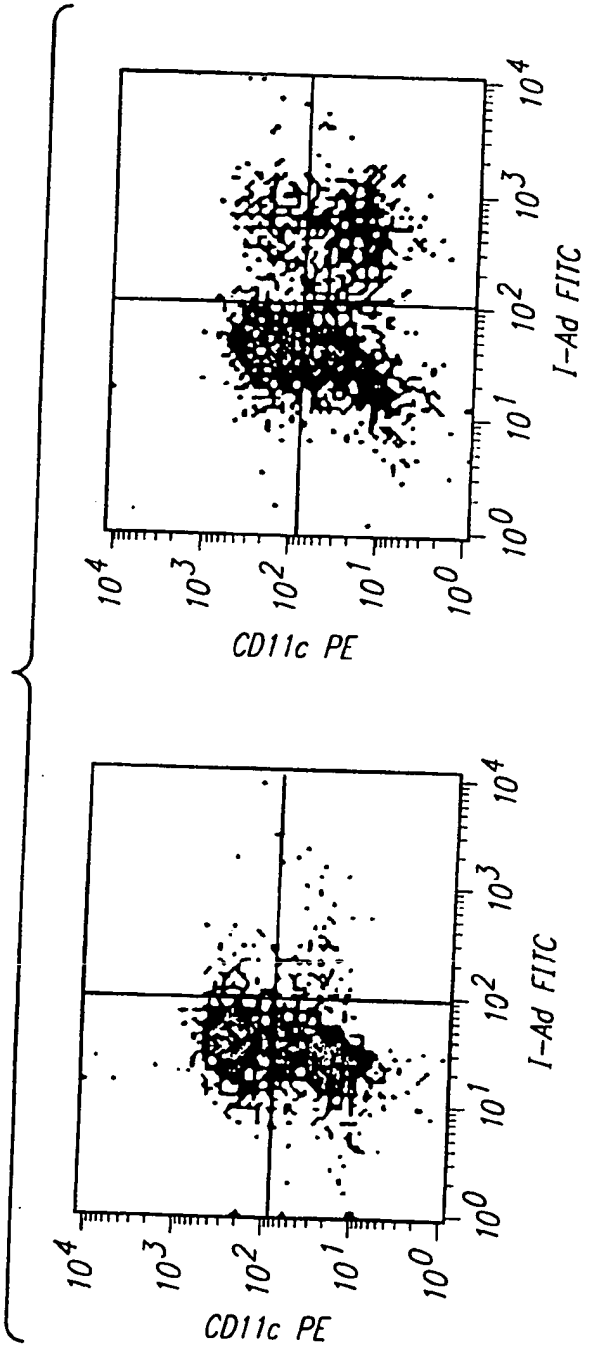


FIG. 5C

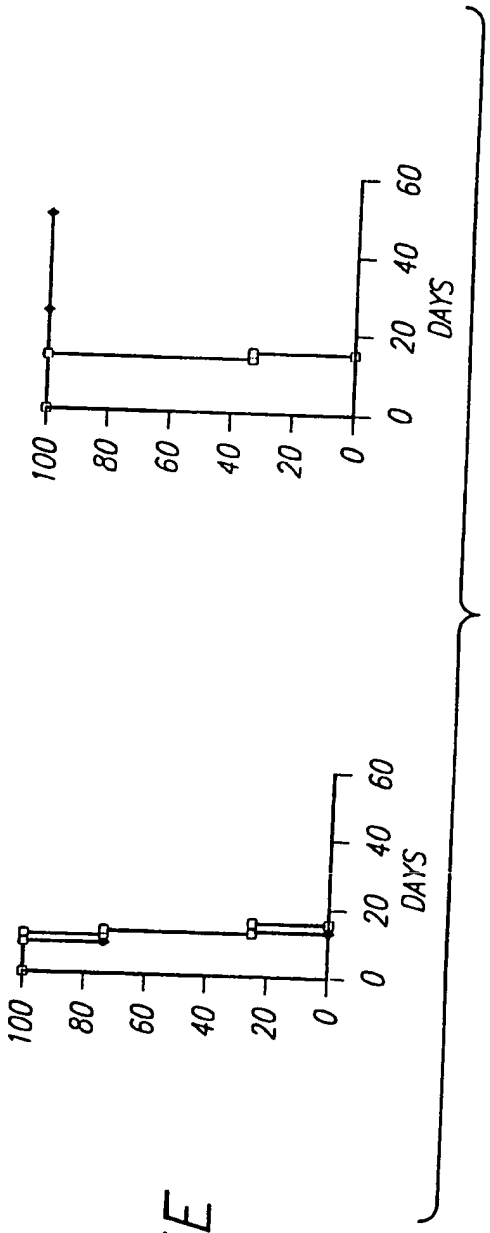
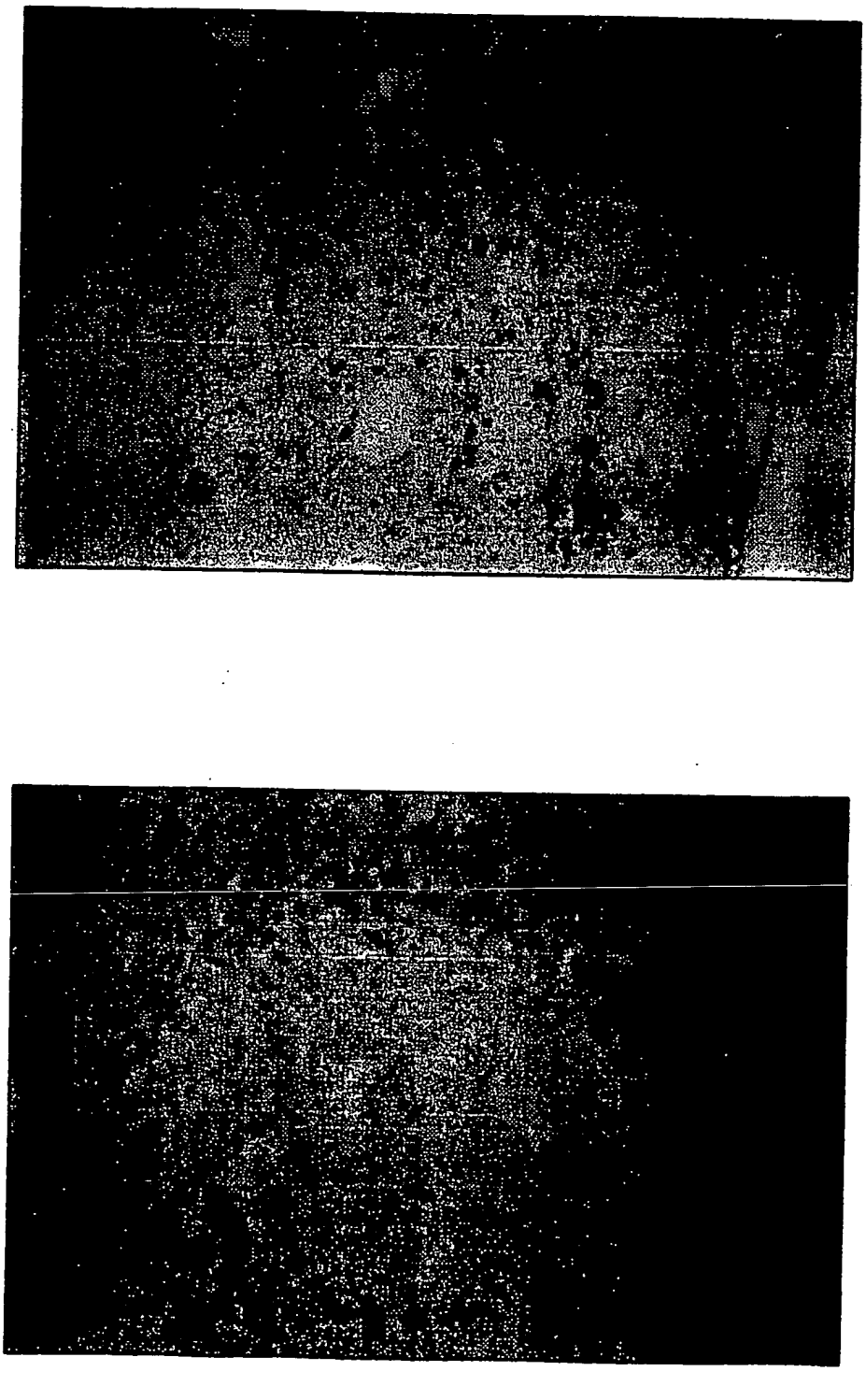


FIG. 5E

FIG. 5D



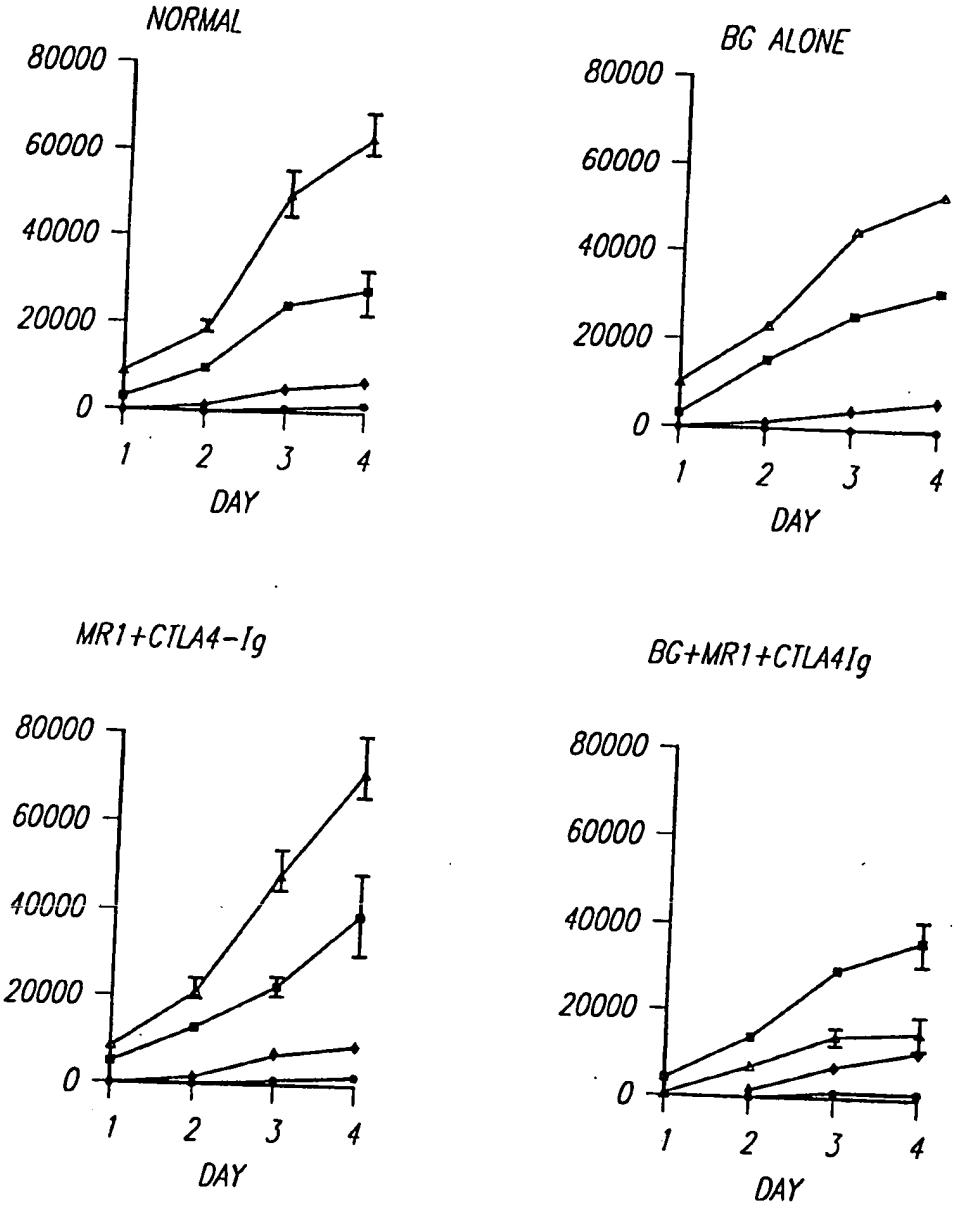
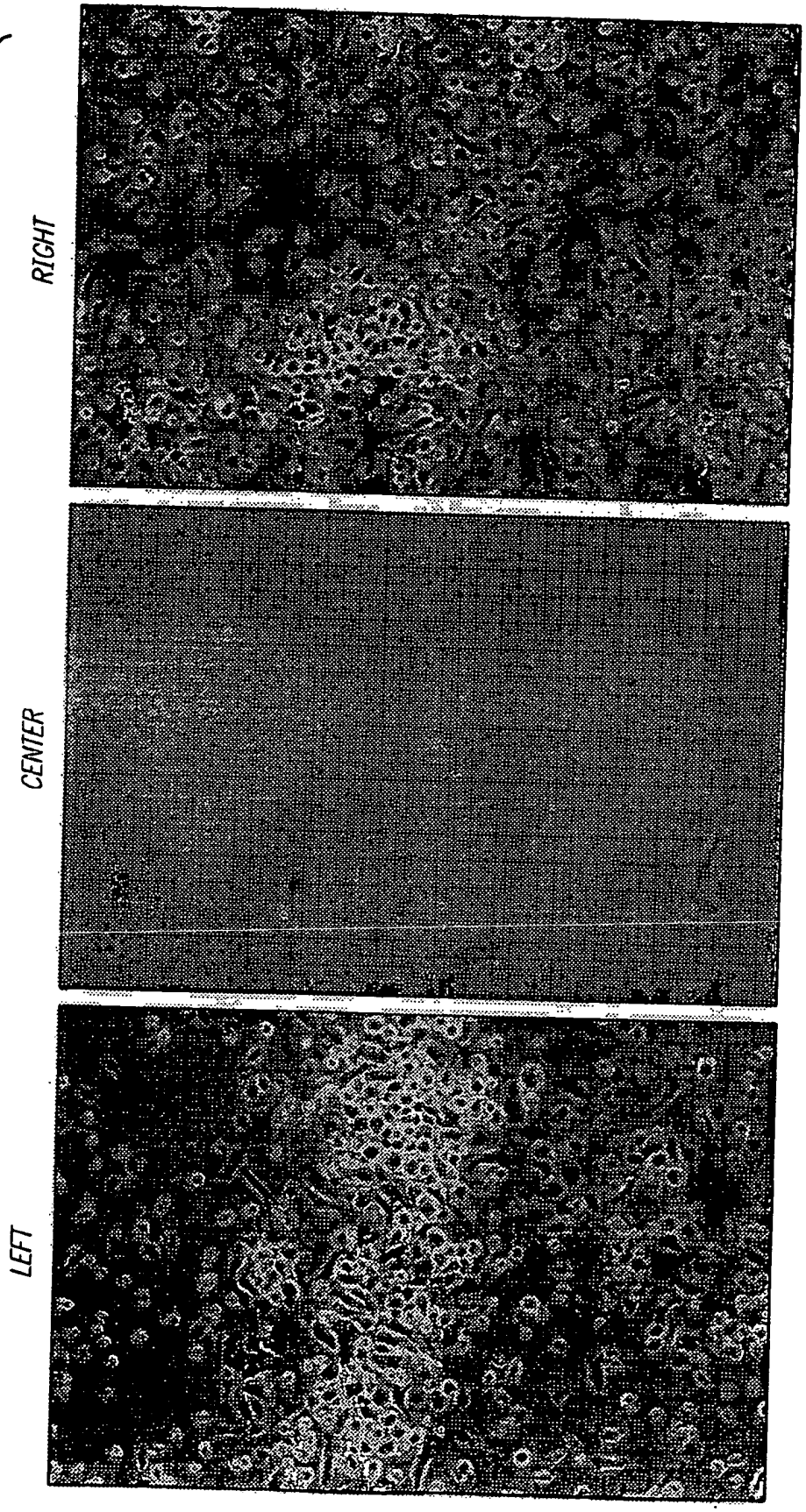

NORMAL
80000
60000
40000
20000
0
1
2
3
4
DAY
BG ALONE
80000
60000
40000
20000
0
1
2
3
4
DAY
MR1+CTLA4-Ig
80000
60000
40000
20000
0
1
2
3
4
DAY
BG+MR1+CTLA4Ig
80000
60000
40000
20000
0
1
2
3
4
DAY

FIG. 6A

FIG. 6B



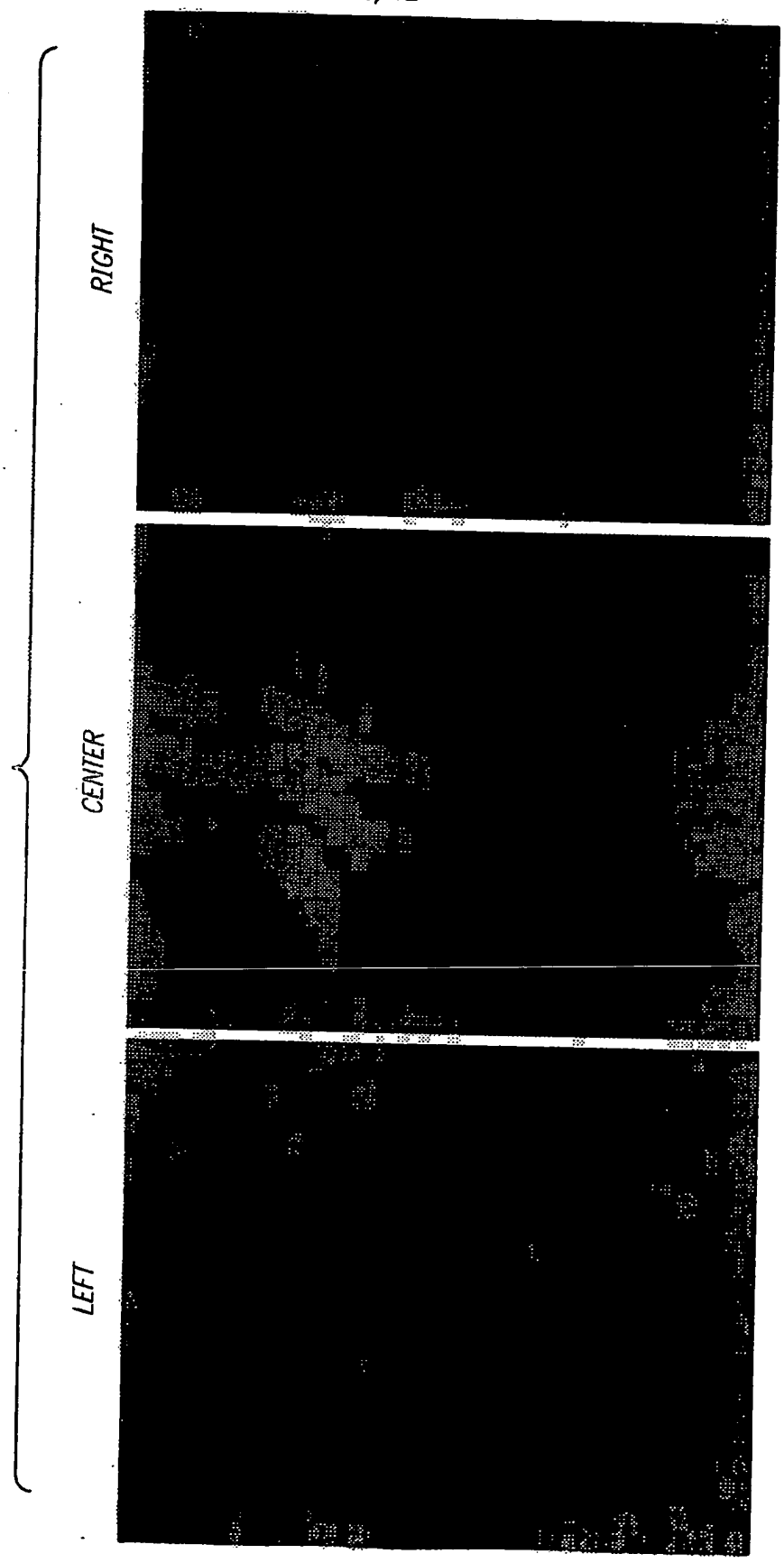


FIG. 6C

FIG. 6D

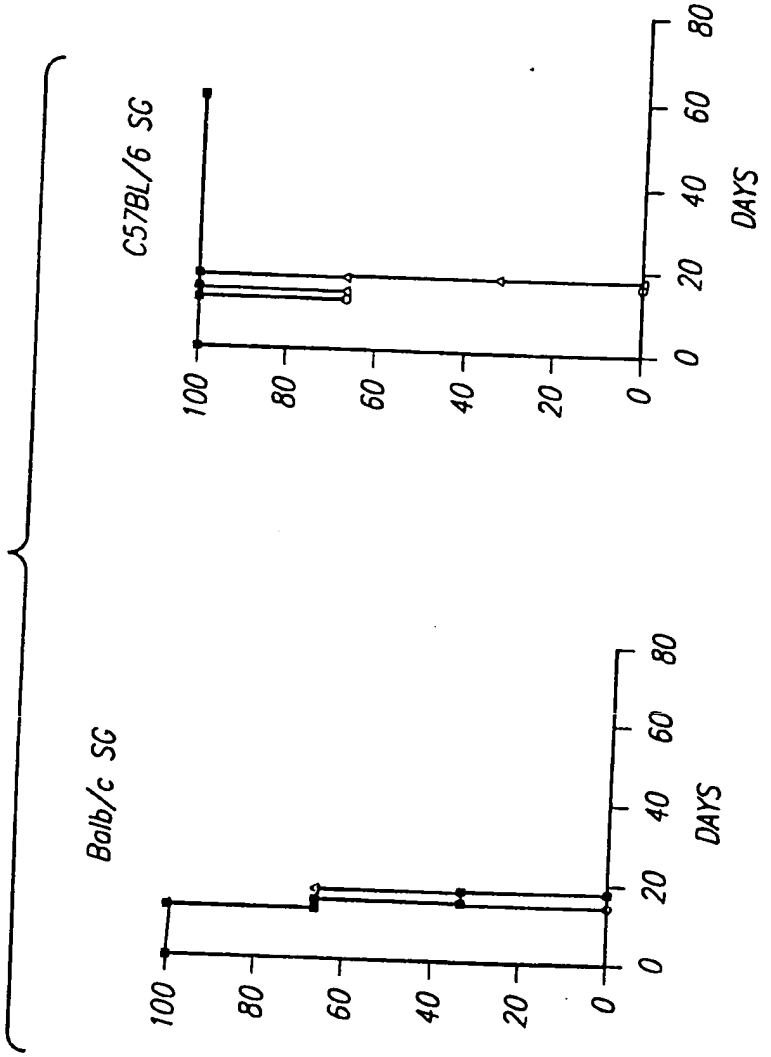
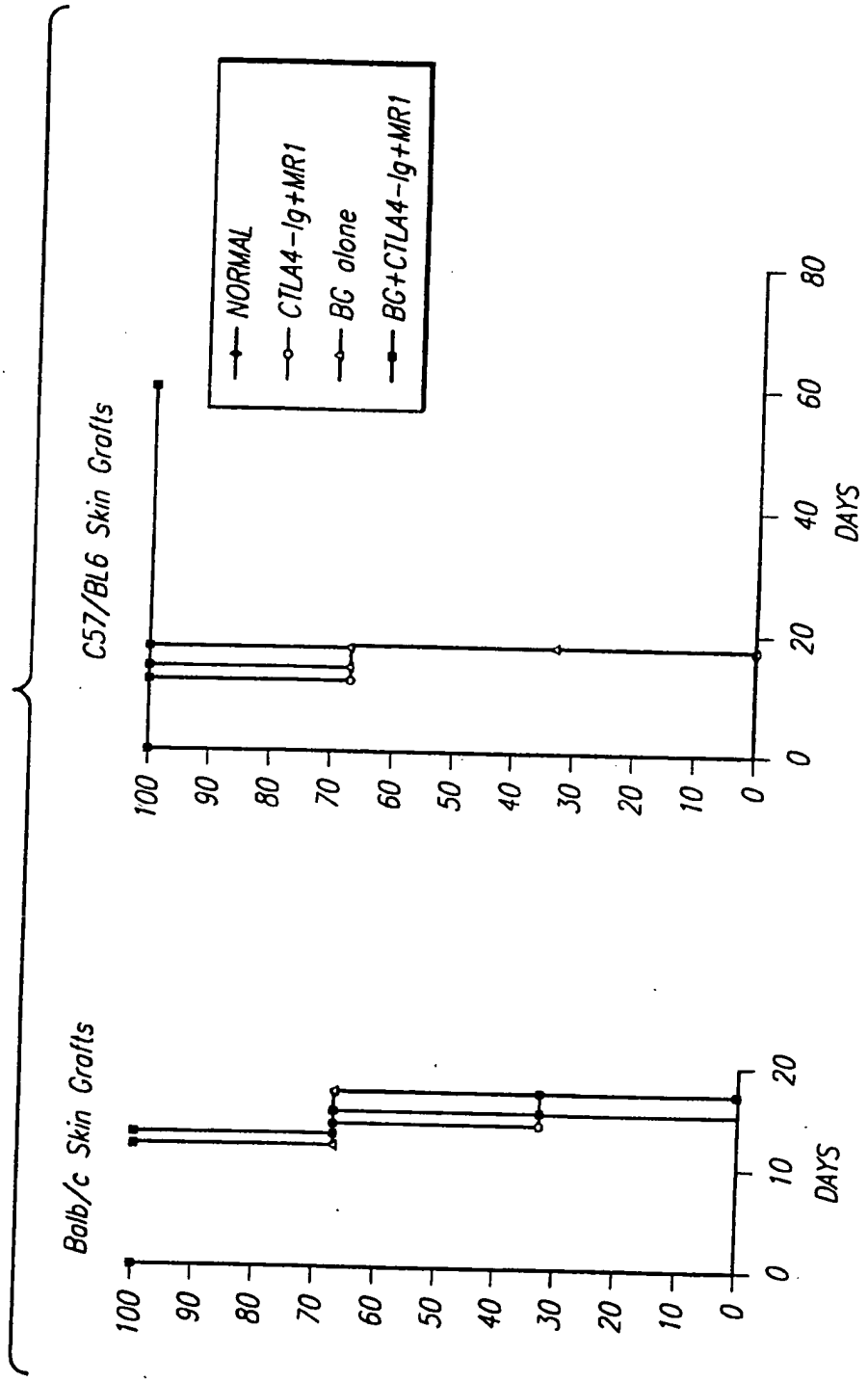


FIG. 7


C57/BL6 Skin Grafts
Balb/c Skin Grafts
NORMAL
CTLA4-Ig+MR1
BC alone
BC+CTLA4-Ig+MR1
DAYS
DAYS
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80

INTERNATIONAL SEARCH REPORT

Interi ɔnal Application No
PCT/US 98/18866

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L27/00 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 405 390 A (O'LEARY ROBERT K ET AL) 11 April 1995 see claims 1,12 see page 5, column 6, line 19 - line 63 ---	1
X	WO 96 38543 A (DIACRIN INC) 5 December 1996 see page 25, line 6 - line 10 see page 37, line 28 - line 37 ---	1
X	HISHA H ET AL: "Successful bone marrow transplantation by bone grafts in chimeric-resistant combination" EXPERIMENTAL HEMATOLOGY, vol. 23, no. 4, April 1995, pages 347-352, XP002091494 see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 February 1999	18/02/1999

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ...nal Application No
PCT/US 98/18866

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 01534 A (UNIV PITTSBURGH) 20 January 1994 see the whole document	1
A	US 5 580 781 A (NAUGHTON GAIL K ET AL) 3 December 1996 see page 29, column 3, line 45 - line 50 see page 35, column 15, line 45 - line 55 see page 35, column 16, line 16 - line 25 see page 36, column 18, line 23 - line 36	1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 18866

Box I Observati ns where certain claims were found unsearchable (Continuation f item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 2-25
because they relate to subject matter not required to be searched by this Authority, namely:
Although claim is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
Rule 39.1(iv) PCT-Method for treatment of the human/animal body by surgery

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee. this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant. this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ...nal Application No
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